Structure-specific detection of plant cuticle bound residues of chlorothalonil by ELISA†

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Abstract: Monoclonal antibodies (mAb) against the main photo-addition products of chlorothalonil with olefinic compounds of plant cuticles were produced. An indirect competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of free and bound chlorothalonil and its derivatives. For the characterization of the binding properties of the mAb, derivatives of chlorothalonil (simulating structures of cuticle bound residues) were synthesized. The cross-reactivities of these products were determined by ELISA. The test system was employed to detect bound residues of chlorothalonil in enzymatically isolated tomato cuticles, which had been spiked with methanolic solutions of the compound, irradiated by simulated sunlight and extracted. The use of isolated cuticles allows work to be carried out with authentic material without disturbance by metabolic processes.

Keywords: chlorothalonil; ELISA; cross-reactivity; cuticle; bound residues

1 INTRODUCTION

Bound residues of pesticides in plants are formed by various mechanisms. For example, pesticides may be conjugated to amino acids or carbohydrates directly or after metabolic activation, and incorporated into macromolecules like proteins, lignin or polysaccharides. Another route for the formation of bound residues is by photochemical activation of the pesticide on the plant surface and subsequent binding to components of the plant cuticle (wax, cutin). In 1984, Schwack was the first to report on the photo-induced formation of reaction products between organic pesticides and constituents of the plant cuticle.[1,2] Using model systems, Schwack *et al* further demonstrated the possibility of addition reactions of different pesticides to cuticle components.[3–5]

In the last 30 years, bound residues have been analysed mainly by the detection of the non-extractable radioactivity after application of ^{14}C-labelled pesticides. However, with this method it is impossible to study the chemical structure or binding sites of bound residues, since binding of pesticides and incorporation of labelled carbon into macromolecules may occur. Consequently, this procedure is not suitable for obtaining information about the possible biological activity of these compounds.

In the last few years, immunochemical methods for the detection of bound residues have been established. Significant research work has been done by Hock *et al* and Niessner *et al* to detect bound residues of atrazin in soil[6,7] and plant tissue.[8]

Chlorothalonil (tetrachloroisophthalonitrile; Fig 1, **1**) is a widely used non-systemic fungicide with protective action. Our earlier experiments have shown that bound residues of **1** are formed in plant cuticles exposed to sunlight.[9] Until now, these residues have only been detectable by the increasing concentration of pesticide-specific elements (Cl, N) in the cuticle. In this paper the structure-specific detection of bound residues by monoclonal antibodies (mAb) against the main photoaddition products of **1** with olefinic compounds of plant cuticles (eg cutin acids, terpenes) (Fig 1) is reported.

2 MATERIALS AND METHODS

2.1 Reagents and equipment

All reagents were of analytical grade unless specified otherwise. MPL® (monophosphoryl lipid A) + TDM (synthetic trehalose dicorynomycolate) emulsion R-700 was purchased from Ade Laborbedarf (Munich, Germany). Cyclohexane, dibenzoyl peroxide, dichloromethane, potassium fluoride, ethylene glycol, ethanolamine, methylene chloride, trimethyliodosilane, ethylene diamine, molecular sieve UOP type 4Å and anhydrous glycerol for molecular biology were purchased from Fluka (Deisenhofen, Germany). Acetonitrile, sodium citrate, sodium azide, *N,N*-

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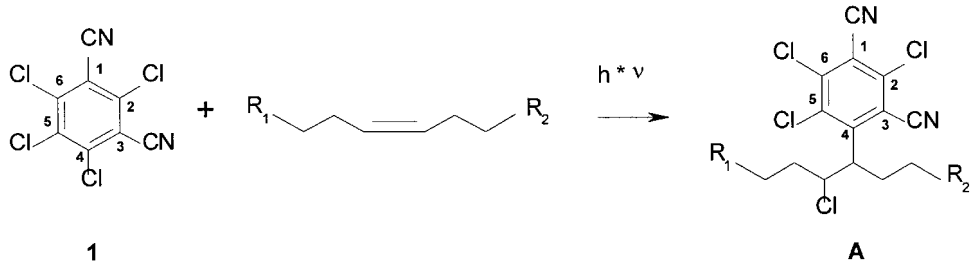


Figure 1. Suggested photochemical binding mechanism of chlorothalonil (**1**) with olefinic compounds of plant cuticles.[9]

dimethylformamide, butylmercaptan, methanol, hexane, diethyl ether and petroleum ether (boiling range 40–60 °C) were purchased from Merck (Darmstadt, Germany). All solvents were distilled before use. Pectinase was obtained from ICN (Costa Mesa, USA). Cellulase was purchased from Roth (Karlsruhe, Germany). 1,3-Dicyanobenzene, BSA (bovine serum albumin, fraction V powder, minimum 98%), Tween® 20, hemocyanin (limulus polyphemus hemolymph type VIII, 95% protein), SIGMA *FAST*™ (*p*-nitrophenyl phosphate tablet set, 20 ml) and 1,1′-carbonyldiimidazole were obtained from Sigma-Aldrich (Steinheim, Germany). Myeloma cell line P3X63Ag8U.1 was obtained from American Type Culture Collection (ATCC).

Water was purified by a Milli-Q 185 plus water purification system (Millipore Corporation, Bedford, USA).

Chlorothalonil was purified from Daconil® (ISK Biotech, Mentor, USA): Daconil® (5 g) was added to acetone (500 ml). The solution was stirred for a few minutes, filtered and the solvent distilled off. The residue was purified by recrystallization from acetone.

Secondary antibody (alkaline phosphatase-conjugated affinity purified goat anti-mouse IgG (H+L), minimal cross-reaction to human, bovine and horse serum proteins) was purchased from Jackson ImmunoResearch Laboratories (West Grove, USA).

Washing buffer: phosphate-buffered saline (PBS) (pH 7.4) containing 0.5 ml litre^{-1} Tween® 20.

ELISA solution: 120 mmol (7.013 g) sodium chloride, 2.7 mmol (201 mg) potassium chloride, 1.97 mmol (268 mg) potassium dihydrogen phosphate, 8.03 mmol (1.43 g) disodium phosphate, 3.08 mmol (200 mg) sodium azide, 2 g BSA and 1 ml Tween® 20 were made up with water to 100 ml in a volumetric flask. The pH was adjusted with 0.1 M sodium hydroxide to pH 7.4 and the solution diluted by water (1+9 by volume) before use.

2.2 General methods

2.2.1 AOX (adsorbable organic halogen)

This was measured using a pyrolysis-microcoulombmeter-apparatus (Euroglas, Delft, Netherlands); oven temperature: 1050 °C; O_2 flow-rate through oven: 150–200 ml min^{-1}; O_2 flow-rate past oven: 40 ml min^{-1}; electrolyte 75% acetic acid with 5 g litre^{-1} sodium chlorate and 5 g litre^{-1} amidosulfuric acid.

2.2.2 UV spectra

These were measured with a Varian Cary 1E spectrophotometer (Darmstadt, Germany).

2.2.3 [^{1}H] and [^{13}C]NMR spectra

These spectra were recorded on Bruker (Karlsruhe, Germany) ARX-500 spectrometers at 500 and 126 MHz, respectively, at a temperature of 300 K, unless specified otherwise. Chemical shifts (δ) are given in ppm relative to internal tetramethylsilane; s=singlet, d=doublet, t=triplet, q=quartet, qui=quintet, sex=sextet, m=multiplet, bt=broad triplet, nr=not resolved; with dt, tt, ddd and dddd denoting a combination.

2.2.4 GLC-MS

This was performed on a Finnigan (Bremen, Germany) MAT Ion Trap 800 equipped with a Perkin-Elmer 8420 gas chromatograph (Perkin-Elmer, Überlingen, Germany). Injector and transfer line temperatures were set at 270 °C. Injection was in the split mode into a fused silica capillary column (0.25 mm × 30 m) wall-coated with PVMS 54 (0.3 μm film thickness, Perkin-Elmer), programmed from 100 to 300 °C at 8 °C min^{-1} with 20 min isothermal at 300 °C. Helium was used as carrier gas.

2.2.5 HPLC-AP(neg)cI-MS

Values were obtained with a model 140b gradient pump (Applied Biosystems, Germany) coupled to a TSQ 700 (Finnigan) mass spectrometer with an APcI interface. Column: Eurospher 100-C18 (Knauer, Berlin, Germany) reversed-phase (250 × 4 mm), 5 μm; guard column (10 × 4 mm), 5 μm; flow rate: 0.8 ml min^{-1}, methanol - formate buffer (0.01 M, pH 4.0) gradient: 60 (0)–60 (5)–100 (25)–100 (35)–60 (40)–60 (45)% methanol (t [min]). DAD trace: 234 nm.

2.2.6 Preparative HPLC

A Kronlab (Sinsheim, Germany) sunflow 100 liquid chromatograph was employed, combined with a Kronlab variable wavelength monitor (detection wavelength: 234 nm) and a Kronlab HPLC column (guard column 20 × 50 mm, column 20 × 250 mm; Nucleosil RP18, 7 μm; flow rate: 20 ml min^{-1}).

2.2.7 Preparative thin-layer chromatography
Coated glass plates, 20 × 20 cm silica gel 60 F_{245} (film thickness 2 mm; Merck) were used.

2.2.8 ELISA
This was performed in microtiter plates (Nunc-Immuno Plate MaxiSorp F96, Denmark) covered between work processes by adhesive plate sealers. Incubation steps and colour development were performed on a M-1000 Microplate Shaker (MedTec Inc, USA). Washing procedures were done by an eight-channel Microplate Washer (MultiWash PLUS™, Tri-Continent, USA). Absorbance was measured by a Microplate Reader EL800, (Bio-Tek Instruments, USA) (software: MikroWin Version 3.0, Mikrotek Laborsysteme GmbH, Germany).

2.2.9 Irradiation
A suntest CPS+ (Heraeus-Industrietechnik, Kleinostheim, Germany) was employed.

2.2.10 Melting points
These were determined on a digital melting point apparatus model 8100 (Electrothermal Eng Ltd, UK).

2.3 Syntheses

The substitution pattern of the chlorothalonil derivatives prepared is given in fig 2. The compounds were synthesized as described below, and supporting spectral, MS and NMR data are given in Appendix 1.

*2.3.1 2,4,5-Trichloro-6-(2-hydroxyethoxy)isophthalonitrile (**2**)*
Based on a synthesis described by Heilmann *et al*,[10] **1** (1.9 mmol, 500 mg) and potassium fluoride (4.7 mmol, 273 mg) were dissolved in ethylene glycol (20 ml) and stirred at 90 °C for 24 h. After cooling to room temperature, the solution was poured on ice (50 g) to induce precipitation. The precipitate was separated, washed with water and air-dried. **2** was isolated by preparative HPLC (methanol + water, 50 + 50 by volume, isocratic) and preparative TLC (diethyl ether + petroleum ether, 50 + 50 by volume; elution from the isolated zone with diethyl ether); yield: 240 mg (43.8%).

*2.3.2 tert-Butyl N-(2-hydroxyethyl)-N-(2,3,5-trichloro-4,6-dicyanophenyl)carbamate (**3**)*
Based on a synthesis described by Heilmann *et al*,[10] **1** (1.9 mmol, 500 mg) and potassium fluoride (3.9 mmol, 227 mg) were dissolved in *N*-(*tert*-butoxycarbonyl)-2-aminoethanol (3.5 g, prepared following a procedure by Bersch *et al*[11]) and stirred at 90 °C for 12 h. After cooling to room temperature, the mixture was poured into water (10 ml) and extracted with dichloromethane (25 ml). The extract was washed with water (2 × 10 ml) and the solvent removed. **3** was isolated by preparative HPLC (methanol + water, 80 + 20 by volume, isocratic) and preparative TLC (diethyl ether + petroleum ether, 55 + 45 by volume; elution from the isolated zone by diethyl ether); yield: 50 mg (7%).

*2.3.3 2,4,5-Trichloro-6-[(2-hydroxyethyl)amino]isophthalonitrile (**4**)*
To a solution of **3** (0.05 mmol, 18 mg) in dichloromethane (700 µl), trimethyliodosilane (0.4 mmol, 50 µl) and, after 1 min, methanol (200 µl) were added. The solvent was removed and the residue taken up in methanol. **4** was isolated by preparative HPLC (methanol + water, 80 + 20 by volume, isocratic); yield: 12 mg (90%).

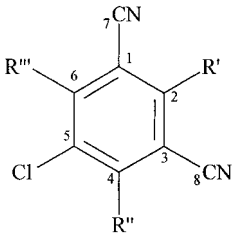
*2.3.4 4-[(2-Aminoethyl)amino]-2,5,6-trichloroisophthalonitrile (**5**)*
Chlorothalonil (0.4 mmol, 100 mg) was dissolved in dichloromethane (20 ml). After addition of a solution of ethylene diamine in water (4 + 20 by volume) the mixture was stirred for 24 h at room temperature. The dichloromethane layer was washed with water (3 × 10 ml) and the solvent was removed. From the residue dissolved in methanol (10 ml), **5** was isolated by preparative HPLC (methanol + 0.01 M formate buffer, 60 + 40 by volume, isocratic); yield: 40 mg (37%).

*2.3.5 4-(Butylsulfanyl)-2,5,6-trichloroisophthalonitrile (**6**) and 4,6-di(butylsulfanyl)-2,5-dichloroisophthalonitrile (7)*
Chlorothalonil (0.4 mmol, 100 mg) and potassium fluoride (0.8 mmol, 45 mg) were dissolved in methanol (10 ml), butanethiol (10 ml) was added, and the solution stirred at room temperature for 12 h. **6** and **7** were isolated by preparative HPLC (methanol + water, 80 + 20 by volume, isocratic); yields: 41 mg of **6** (34%), 19 mg of **7** (14%).

*2.3.6 2,4,5-Trichloro-6-cyclohexylisophthalonitrile (**8**), 4,5,6-trichloro-2-cyclohexylisophthalonitrile (**9**), 2,5-dichloro-4,6-dicyclohexylisophthalonitrile (**10**), 4,5-dichloro-2,6-dicyclohexylisophthalonitrile (**11**), 5-chloro-2,4,6-tricyclohexylisophthalonitrile (**12**) and 2,4,5-trichloro-6-(1-methylpentyl)isophthalonitrile (**13**)*
A solution of **1** and dibenzoylperoxide (DBP) in 40 ml cyclohexane (for products **8–12**) or *n*-hexane (for product **13**) was heated under reflux. The solvent was removed and the residue dissolved in methanol (10 ml). The compounds were isolated by preparative HPLC and preparative TLC (elution from the isolated zone by diethyl ether). Conditions for synthesis and product isolation are given in Table 1; yields: 60 mg of **8** (19%), 15 mg of **9** (5%), 25 mg of **10** (14%), 25 mg of **11** (14%), 20 mg of **12** (10%), 15 mg of **13** (5%).

*2.3.7 2,4,5-Trichloroisophthalonitrile (**14**) and 2,5-dichloroisophthalonitrile (**15**)*
These were prepared as described by Zorn.[9]

*2.3.8 2,4,5-Trichloro-6-hydroxyisophthalonitrile (**16**)*
This compound was synthesized following the method of Heilmann *et al.*[10]



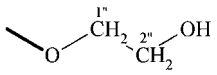
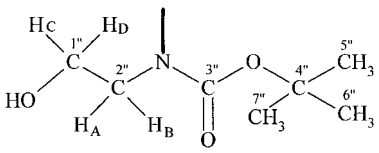
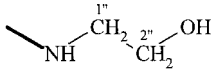
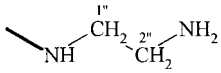
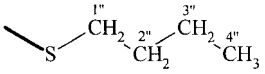
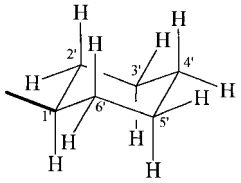
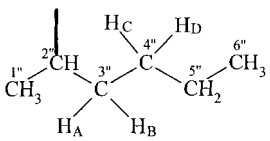
	R'	R''		R'''
1	-Cl	-Cl		-Cl
2	-Cl	$-C_2H_5O_2$		-Cl
3	-Cl	$-C_7H_{14}NO_3$		-Cl
4	-Cl	$-C_2H_6NO$		-Cl
5	-Cl	$-C_2H_7N_2$		-Cl
6	-Cl	$-C_4H_9S$		-Cl
7	-Cl	$-C_4H_9S$		$-C_4H_9S$
8	-Cl	$-C_6H_{11}$		-Cl
9	$-C_6H_{11}$	-Cl		-Cl
10	-Cl	$-C_6H_{11}$		$-C_6H_{11}$
11	$-C_6H_{11}$	$-C_6H_{11}$		-Cl
12	$-C_6H_{11}$	$-C_6H_{11}$		$-C_6H_{11}$
13	-Cl	$-C_6H_{13}$		-Cl
14	-Cl	-H		-Cl
15	-Cl	-H		-H
16	-Cl	-OH		-Cl

Figure 2. Substitution pattern of synthetic chlorothalonil derivatives.

Table 1. Conditions for the syntheses of compounds **8–13**

Compound	Chlorothalonil initial weight (mmol)	(mg)	DBP initial weight (mmol)	(mg)	Heating time (h)	Eluent composition HPLC ($MeOH + H_2O$) (by volume), isocratic	Eluent composition TLC ($DE^a + PE^b$) (by volume)
8	1	266	1	242	3	90+10	10+90
9	1	266	1	242	3	90+10	10+90
10	0.5	133	2	484	5	95+5	5+95
11	0.5	133	2	484	5	95+5	5+95
12	0.5	133	2.5	606	18	95+5	5+95
13	1	266	1	242	8	85+15	–

[a] DE: diethyl ether.
[b] PE: petroleum ether.

2.4 Preparation of immunogen (hemocyanin-2 conjugate) and coating antigen (BSA-2 conjugate)

Product **2** (16.3 mg, 56 μmol) was dissolved in DMF (2.0 ml) and 1,1′-carbonyldiimidazole (9.1 mg, 56 μmol) was added. The solution was stirred for 3 h and slowly added to a solution of hemocyanin (39.1 mg) or BSA (37.7 mg), respectively, in PBS (6 ml, adjusted to pH 10.0). The conjugate was dialysed (8000 D) against PBS (1000 ml, pH 7.4), followed by water (1000 ml).

Yields after lyophilisation: 14.0 mg immunogen, 17.3 μg Cl mg^{-1} conjugate (analysis of AOX) corresponding to a molar conjugation ratio of 11:1 (**2**:hemocyanin); 35.5 mg coating antigen, 8.5 μg Cl mg^{-1} conjugate (analysis of AOX) corresponding to a molar conjugation ratio of 5:1 (**2**:BSA).[9]

2.5 Immunization and production of mAb

BALB/c mice were immunized three times (days 1, 8, 25) intraperitoneally with an emulsion consisting of 1.1 mg hemocyanin-**2** conjugate in 20 μl water and 200 μl MPL® + TDM adjuvant. Three days after the last injection, mice were killed and the spleen cells were fused with the myeloma cell line P3X63Ag8U. 1.[12] Screening for antibody-producing hybridomas was performed by the dot test as described by Hawkes *et al.*[13] For this purpose 4 μg hemocyanin-**2** conjugate and BSA-**2** conjugate each were dissolved in 0.8 μl water and dropped onto a nitrocellulose sheet. The fluid was allowed to evaporate.

The hybridoma supernatants were diluted by glycerol (1 + 1 by volume) and stored at −18 °C until use (**mAb Chl. 4/11**).

2.6 Sample preparation

2.6.1 *Isolation of tomato cuticles*

Tomato cuticles were enzymatically isolated as described by Riederer and Schönherr,[14] modified by Schynowski.[15] Tomato fruits grown without the use of pesticides and free of visible defects were selected for cuticle isolation. Cuticles were isolated with an aqueous mixture of pectinase (20 g $litre^{-1}$), cellulase (2 g $litre^{-1}$) and NaN_3 (1 mmol) in sodium citrate buffer (50 mmol; pH 3,5) for 48 h at 37 °C. After the cuticular membrane was separated, any adhering debris was removed with a jet of water. The isolated membrane was then extensively washed with water and air-dried.

2.6.2 *Treatment of cuticles*

Isolated cuticles were dipped into methanolic solutions of **1** (1 g $litre^{-1}$), air-dried and divided into two parts of similar size. One part was irradiated with simulated sunlight for 8 h (**I**). Meanwhile, the other part was stored in the dark (**D**). In order to exclude non-specific binding of mAb to the cuticles, the procedure was repeated simultaneously without dipping cuticles into the methanolic solution of **1** (cuticles not dipped and irradiated: $\mathbf{I_{nd}}$; cuticles not dipped and stored in the dark: $\mathbf{D_{nd}}$). The cuticles were exhaustively extracted with hot acetone in a Soxhlet apparatus (twice, 4 h each).

2.7 Development of an indirect competitive ELISA

2.7.1 *Titer analysis*

The titer of mAb was determined by checkerboard titration (two-dimensional titration method) measuring the binding of serial dilutions (1:500 to 1:8000) coated with several concentrations of BSA-**2** conjugate (2.5 to 0.05 mg $litre^{-1}$). Optimum concentrations for coating antigen and mAb dilution were determined (0.5 mg $litre^{-1}$, 1:2000).

2.7.2 *ELISA procedure*

For *coating*, 250 μl of 0.5 mg $litre^{-1}$ BSA-**2** conjugate in water were placed in each well of a microplate. After incubation overnight at 25 °C, the plates were washed four times.

For *blocking*, 250 μl of 10 g $litre^{-1}$ BSA in ELISA solution were added to all wells, which were incubated overnight at 25 °C and washed four times. The plates were stored at −18 °C and equilibrated at room temperature for 30 min prior to use.

The *preincubation* was performed for 2 h at 25 °C on an orbital shaker in sealed tubes (10 ml): 4.85 ml ELISA solution and 50 μl methanol (A_0), 50 μl $\mathbf{1}_{excess}$ solution (1 mg ml^{-1} **1** in methanol) (A_{excess}) or 50 μl methanolic standard solution were added to each tube. Finally, 100 μl of mAb (diluted 1 + 39 by volume with ELISA solution) were added, resulting in a total volume of 5.0 ml. For the analysis of tomato cuticles,

the preincubation step was performed as described in Section 2.7.3.

Incubation I was carried out for 1 h at 25 °C: 200 µl preincubated solutions were added to each well (at least three replicates). The plates were washed four times.

Incubation II was performed for 2 h at 25 °C: 200 µl of an alkaline phosphatase conjugated secondary antibody (diluted 1 + 5000 by volume with ELISA solution) was placed in each well. The plates were washed four times.

Colour development and measurement: 200 µl SIGMA *FAST*™ solution was added to all wells. The absorbance was measured immediately and after colour development for 1 h at 25 °C.

2.7.3 Detection of bound residues in plant cuticles

Five milligrams of the cuticles (**I, D**, $\mathbf{I_{nd}}$, $\mathbf{D_{nd}}$) each were placed in sealed tubes (10 ml). ELISA solution (4.85 ml) methanol (50 µl) and mAb (100 µl, diluted 1 + 39 by volume with ELISA solution) were added to each flask. These samples were preincubated for 2 h at 25 °C. Additionally, A_{excess} and A_0 samples were treated as described above. Aliquots of each sample were placed in six wells.

The given procedure was repeated with preceding preincubation overnight ($\mathbf{I_{on}}$, $\mathbf{D_{on}}$). As non-specific binding to cuticles did not occur, undipped samples were not repeated.

3 RESULTS AND DISCUSSION

3.1 Syntheses

For characterization of binding properties of the mAb, derivatives of **1** (simulating possible structures of cuticle bound residues) were synthesized. Derivatives of **1** substituted via heteroatoms (**2–7**) were produced by nucleophilic substitution with an alcohol, amine or mercaptan, based on a synthesis described by Heilmann *et al.*[10] Since mono-, di- and trialkylated derivatives of **1** have not been described in literature so far, it was essentially necessary to develop novel syntheses to obtain these structures as model and test compounds for photochemically bound plant cuticle residues. For the electron-poor aromatic system of **1**, a free-radical addition process initiated by dibenzoyl peroxide turned out to be an effective pathway. By this procedure **8–13** were synthesized. Figure 2 shows the substitution pattern of all synthesized chlorothalonil derivatives. Compounds **3–13** are described for the first time. Compounds **14** and **15** are products of **1**, isolated after irradiation of **1** in cyclohexane.[9] Compound **16** could be detected in metabolism studies of **1** in some plants in orders of magnitude of about 2%.[16]

3.2 ELISA evaluation method

For evaluation of the ELISA, a two-parameter model was chosen, which in well-functioning assays is as good as the four-parameter fit. The delta absorbances were used, checked for outliers with a Gauss test (level of significance: 95%) and $\%B/B_0$-values were calculated.[17]

$$\%B/B_0 = \frac{A - A_{excess}}{A_0 - A_{excess}} \times 100$$

A_0, the upper asymptote of the curve = 100%

A_{excess}, the lower asymptote = 0%

An example for a calibration curve of **1** is given in Fig 3.

Linearization of the calibration curves is useful for the evaluation of unknown concentrations. Therefore, for the determination of cross-reactivities (between 20% B/B_0 and 80% B/B_0 corresponding to logit ($\%B/B_0$) between −1.39 and 1.39), linearization is

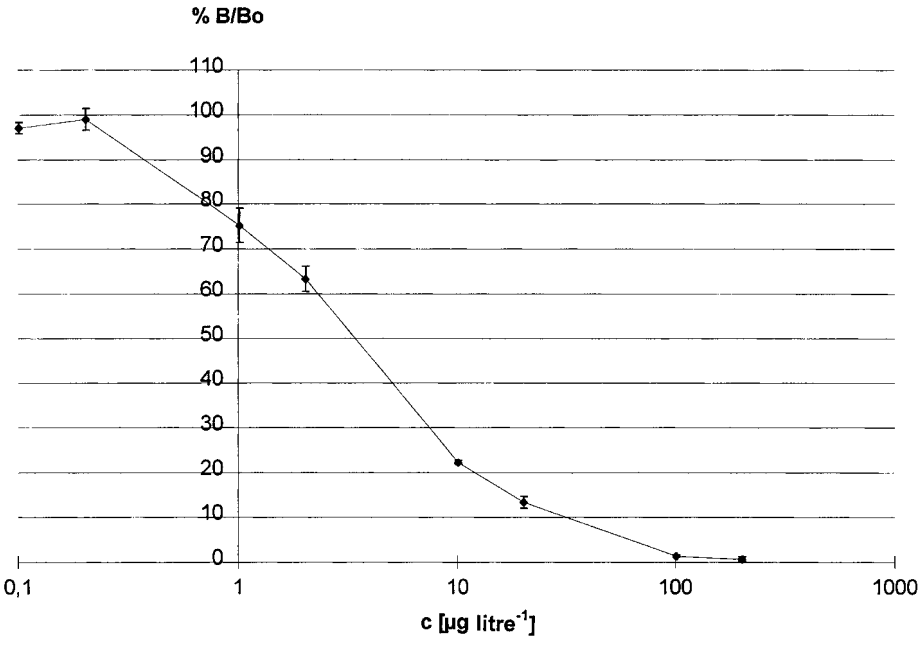


Figure 3. Calibration curve of chlorothalonil (mean ± SD).

Table 2. Cross-reactivities (IC_{50}) of mAb

Substance	*Cross-reactivity (IC_{50})*[a]
1	100
2	72.7
3	2.0
4	155.1
5	293.6
6	3.6
7	0.1
8	0.1
9	0.2
10	n d (<0.1)
11	n d (<0.1)
12	n d (<0.1)
13	0.2
14	28.7
15	3.2
16	0.9

[a] Related to molecular ratio.

obtained using the logit-log transformation:

$$\text{logit}(\%B/B_0) = \ln\left(\frac{\%B/B_0}{100 - \%B/B_0}\right)$$

(assay performance: ($\%B/B_0 = 20\%$: 11.0 μg litre^{-1}, $\%B/B_0 = 50\%$: 2.7 μg litre^{-1}, $\%B/B_0 = 80\%$: 0.7 μg litre^{-1})

3.3 Determination of cross-reactivities (IC_{50})

For the determination of cross-reactivities of structurally related compounds, methanolic stock solutions were prepared with concentrations ranging from 200 mg litre^{-1} to 10 μg litre^{-1}. These solutions (50 μl each) were diluted to 5.0 ml, and the concentrations of the calibration solutions thus ranged from 2 mg litre^{-1} to 0.1 μg litre^{-1}. Cross-reactivity (IC_{50}) was calculated according to the formula

% cross-reactivity (IC_{50}) = A/B × 100

A: concentration of **1** at logit ($\%B/B_0$) = 0 ($\%B/B_0 = 50\%$)

B: concentration of the cross-reacting hapten at logit ($\%B/B_0$) = 0 ($\%B/B_0 = 50\%$).[17]

In order to test the precision of the estimation of cross-reactivities, the centre points (logit ($\%B/B_0$) = 0) of four calibration curves of **1** were determined. The coefficient of variation (CV) was 3.9% ($n = 4$, $x_m = 2.74$ μg litre^{-1}).

The results of the determination of cross-reactivities are compiled in Table 2. The cross-reactivities indicate that mAb reacts best with residues bound via a nitrogen bridge in position **4**. The comparison of the cross-reactivities of **3, 4** and **5** clearly shows that a tertiary nitrogen at position **4** causes a cross-reactivity considerably lower than a secondary nitrogen. Therefore, it can be supposed that the affinity of mAb to photo-induced bound residues will be reduced by steric hindrance, since alkyl-substituted **1** derivatives are thought to be the main products of photochemically induced reactions of **1** with olefinic constituents of the plant cuticle (Fig 1). As cross-reactivities of **8** and **13** show, these structures can also be detected by this procedure, but with much lower sensitivity.

Table 3. % B/B_0 values of isolated tomato cuticles

Cuticle sample[a]	*I (% B/B_0)*	*II (% B/B_0)*	*III (% B/B_0)*
I	38.4	39.1	
D	74.7	73.2	
$\mathbf{I_{nd}}$	102.2	98.6	
$\mathbf{D_{nd}}$	98.4	98.7	
$\mathbf{I_{on}}$	30.8	43.4	16.6
$\mathbf{D_{on}}$	79.8	80.8	58.5

[a] **I**: dipped and irradiated, **D**: dipped and stored in the dark, $\mathbf{I_{nd}}$: not dipped and irradiated, $\mathbf{D_{nd}}$: not dipped and stored in the dark, $\mathbf{I_{on}}$: dipped, irradiated and preincubated overnight, $\mathbf{D_{on}}$: dipped, stored in the dark and preincubated overnight.

3.4 Detection of bound residues in plant cuticles

By dipping of isolated tomato cuticles in methanolic solutions of **1**, an initial extractable residue level of the cuticles of about 10 mg g^{-1} was obtained. By enzymatical isolation, 1 kg tomatoes yield about 1.5 g of cuticle. Therefore, the initial residue level of isolated and dipped tomato cuticles corresponded to a residue level of about 15 mg kg^{-1} in tomato fruits. During irradiation for 8 h, the extractable residue level was reduced to about 40% (6 mg kg^{-1}) of the initial content. The difference of 60% (9 mg kg^{-1}) is supposed to be bound to the cuticle and should be detectable by the use of mAb.[9]

Values of $\%B/B_0$ for the detection of bound residues in plant cuticles are given in Table 3. The values of $\mathbf{I_{nd}}$ and $\mathbf{D_{nd}}$ samples demonstrate that non-specific binding of mAb to cuticles does not occur. For dipped cuticles, $\%B/B_0$ values of irradiated samples are significantly smaller than those of dark-stored cuticles. These results indicate that it was possible for the first time to detect photo-induced bound residues of **1** in isolated tomato cuticles by a structure-specific antibody method. Also, bound residues were detectable in dipped and dark-stored cuticles. This effect is probably caused by nucleophilic substitution of **1** by nitrogenous compounds of the plant cuticle.

The use of isolated cuticles allows working with authentic material without disturbance by metabolic processes. As the thickness of tomato cuticles varies with their degree of ripening, each dipped sample was divided into two parts of equal size. One part was irradiated by simulated sunlight, the other part was stored in the dark. With this method, results of **D** and **I** samples can be compared with each other.

4 CONCLUSIONS

For the first time it has been possible to detect bound residues in plant cuticles by ELISA as a structure-specific method. In further studies, fruits from a field experiment and commercial products with extractable contents of **1** will be tested for bound residues. By this

method, it will also be possible to detect any treatment of tomatoes with plant sprays containing **1**, even if extractable **1** cannot be detected. For such investigations, an enhancement of the test sensitivity might be necessary. This should be achievable eg by overnight preincubation. In order to ensure representative sampling, a method for the analysis of an aliquot part of a larger sample size will have to be developed. This should be possible by boron trifluoride-catalyzed depolymerization of cuticles.

Besides the detection of cuticle bound residues the developed ELISA test system could be used in metabolism studies for the determination of extractable chlorothalonil as well as for the control of correct application rates.

Combined with suitable models for gastro-intestinal digestion, this test system could be used to detect released residues in studies on bioavailability.

APPENDIX 1

The hydrogen/carbon numbering used for assignment of spectroscopic data is given in Fig 2. Note that this does not necessarily correspond with the IUPAC numbering of the substituents around the benzene ring.

*Compound **2***

Colourless crystals, melting point 137–139 °C, UV/VIS (acetonitrile) λ_{max} [nm], (log ε): 234 (4.7); 320 (3.3), GLC-MS (70 eV EI): m/z = 290 (44.9%); 292 (43.3%); 294 (14.6%) $[M^{+\cdot}]$/246 (100%); 248 (99.4%); 250 (31.4%) $[C_8\,HCl_3\,N_2\,O^{+\cdot}]$, [^{1}H]NMR ($CDCl_3$): 4.55 (t, 2H, 1″-$H_2$, ^3J(1″-H_2; 2″-H_2): 4.4 Hz), 4.05 (dt, 2H, 2″-H_2), 2.19 (t, 1H, 2″-OH, ^3J(2″-H_2; 2″-OH): 5.9 Hz), [^{13}C]NMR ($CDCl_3$): 161.9 (C-4), 142.5/139.7 (C-2/C-6), 128.0 (C-5), 112.2/111.9/111.7/109.1 (C-1/C-3/C-7/C-8), 78.1 (C-1″), 61.8 (C-2″)

*Compound **3***

Colourless oil, UV/VIS (hexane) λ_{max} [nm], (log ε): 227 (4.6); 292 (3.5); 312 (3.3); 324 (3.3), HPLC-APcI-MS (neg cl): m/z = 388 (100%); 390 (97.8%); 392 (30.4%); 394 (3.3%) $[M-H]^-$, [^{1}H]NMR ($CDCl_3$): 4.05 (ddd, 1H, 2″-H_A, ^2J(2″-H_A; 2″-H_B): (−) 14.6 Hz, ^3J(2″-H_A; 1″-H_C): 3.0 Hz, ^3J(2″-H_A; 1″-H_D): 7.8 Hz), 3.53 (ddd, 1H, 2″-H_B, ^3J(2″-H_B; 1″-H_C): 4.9 Hz, ^3J(2″-H_B; 1″-H_D): 3.2 Hz), 4.00 (ddd, 1H, 1″-H_C, ^2J(1″-H_C; 1″-H_D): (−) 11.2 Hz), 3.83 (ddd, 1H, 1″-H_D), 1.41 (s, 9H, 5″-H_3/6″-H_3/7″-H_3), [^{13}C]NMR ($CDCl_3$): 152.0 (C-3″), 149.0 (C-4), 141.9/138.7 (C-2/C-6), 133.6 (C-5), 116.7/115.6/112.3/111.7 (C-1/C-3/C-7/C-8), 83.7 (C-4″), 61.9 (C-1″), 51.9 (C-2″), 27.9 (C-5″/C-6″/C-7″)

*Compound **4***

Pale yellow crystals, melting point 190 °C, UV/VIS (methanol) λ_{max} [nm], (log ε): 246 (4.4); 298 (4.2); 354 (3.6), HPLC-APcI-MS (neg cl): m/z = 288 (100%); 290 (94.9%); 292 (25.9%); 294 (3.7%) $[M-H]^-$, [^{1}H]NMR (d_6-DMSO): 7.37 (t, 1H, 1″-NH, ^3J(1″-NH; 1″-H_2): 5.9 Hz), 5.01 (t, 1H, 2″-OH, ^3J(2″-OH; 2″-H_2): 5.3 Hz), 3.84 (q, 2H, 1″-H_2, ^3J(1″-H_2; 2″-H_2): 5.6 Hz), 3.65 (q, 2H, 2″-H_2), [^{13}C]NMR (d_6-DMSO): 150.2 (C-4), 141.5/137.8 (C-2/C-6), 118.9 (C-5), 100.5/93.7 (C-1/C-3), 114.6/113.7 (C-7/C-8), 59.5 (C-2″), 46.6 (C-1″)

*Compound **5***

Pale yellow crystals, melting point 166 °C, UV/VIS (methanol) λ_{max} [nm], (log ε): 246 (4.5); 297 (4.3); 350 (3.7), HPLC-APcI-MS (neg cl): m/z = 287 (97.8%); 289 (100%); 291 (37.3%); 293 (4.4%) $[M-H]^-$, [^{1}H]NMR (d_6-DMSO): 3.77 (t, 2H, 1″-H_2, ^3J(1″-H_2; 2″-H_2): 6.2 Hz), 2.91 (t, 2H, 2″-H_2), [^{13}C]NMR (d_6-DMSO): 150.0 (C-4), 141.5/ 137.7 (C-2/C-6), 119.0 (C-5), 114.8/113.8 (C-7/C-8), 100.3/93.4 (C-1/C-3), 45.7 (C-1″), 40.2 (C-2″)

*Compound **6***

Yellow crystals, melting point 89 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 231 (4.6); 250 (4.1); 265 (3.9); 309 (3.6); 351 (3.7), GLC-MS (methanol-cI): m/z = 319 (100%); 321 (95.3%); 323 (25.6%); 325 (4.7%) $[M+H]^+$, [^{1}H]NMR ($CDCl_3$): 3.21 (t, 2H, 1″-H_2, ^3J(1″-H_2; 2″-H_2): 7.4 Hz), 1.63 (qui, 2H, 2″-H_2, ^3J(2″-H_2; 3″-H_2): 7.4 Hz), 1.47 (sex, 2H, 3″-H_2), 0.94 (t, 3H, 4″-H_3, ^3J(3″-H_2; 4″-H_3): 7.4 Hz), [^{13}C]NMR ($CDCl_3$): 148.0 (C-4), 141.1/139.4/138.0 (C-2/C-5/C-6), 118.9/115.8 (C-1/C-3), 112.9/112.0 (C-7/C-8), 36.5/31.8/21.7 (C-1″/C-2″/C-3″), 13.5 (C-4″)

*Compound **7***

Yellow oil, UV/VIS (hexane) λ_{max} [nm], (log ε): 229 (4.5); 265 (4.1); 304 (3.9); 345 (3.8); 361 (3.7), GLC-MS (methanol-cI): m/z = 373 (100%); 375 (80.7%); 377 (14.7%) $[M+H]^+$, [^{1}H]NMR ($CDCl_3$): 3.18 (t, 4H, 1″-H_2/1‴-H_2, ^3J(1″-H_2; 2″-H_2/1‴-H_2; 2‴-H_2): 7.4 Hz), 1.61, qui, 2″-H_2/2‴-H_2, ^3J(2″-H_2; 3″-H_2/2‴-H_2; 3‴-H_2): 7.4 Hz), 1.47 (sex, 4H, 3″-H_2/3‴-H_2), 0.93 (t, 6H, 4″-H_3/4‴-H_3, ^3J(3″-H_2; 4″-H_3/3‴-H_2; 4‴-H_3): 7.3 Hz), [^{13}C]NMR ($CDCl_3$): 146.3 (C-4/C-6), 143.1/139.2 (C-2/C-5), 119.5 (C-1/C-3), 113.4 (C-7/C-8), 36.4/31.8/21.7 (C-1″/C-2″/C-3″/C-1‴/C-2‴/C-3‴), 13.5 (C-4″/C-4‴)

*Compound **8***

Colourless crystals, melting point 176 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 231 (4.9); 308 (3.2); 320 (3.4), GLC-MS (methanol-cI): m/z = 313 (100%); 315 (94.5%); 317 (33.0%); 319 (1.8%) $[(M+H]^+$, [^{1}H]NMR (323 K, $CDCl_3$): 3.56 (bt, 1H, 1″-H, $^3J_{a,a}$ (1″-H; 2″-H_a/6″-H_a): ≈ 11 Hz), 2.26 (m, 2H, 2″-H_a/6″-H_a), 1.95 (m, 2H, 2″- H_e/6″-H_e), 1.79/1.71 (m/m, 1H/2H, 3″-H_a/4″-H_a/5″-H_a), 1.42 (m, 3H, 3″-H_e/

4″-H_e/5″-H_e), [^{13}C]NMR ($CDCl_3$): 155.4 (C-4), 141.5/140.8 (C-2/C-6), 133.8 (C-5), 114.9/113.6/113.0/112.1 (C-1/C-3/C-7/C-8), 43.5 (C-1″), 29.2 (C-2″/C-6″), 26.6 (C-3″/C-5″), 25.1 (C-4″)

Compound ***9***

Colourless crystals, melting point 216 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 231 (4.8); 306 (3.2); 318 (3.4), GLC-MS (methanol-cI): $m/z = 313$ (100%); 315 (95.4%); 317 (28.4%); 319 (6.4%) $[M+H]^+$, [^{1}H]NMR (323 K, $CDCl_3$): 3.33 (tt, 1H, 1′-H, $^3J_{a,a}$ (1′-H; 2′-H_a/6′-H_a): 12.5 Hz, $^3J_{a,e}$ (1′-H; 2′-H_e/6′-H_e): 3.6 Hz), 2.23 (m, 2H, 2′-H_a/6′-H_a), 1.95 (m, 2H, 2′-H_e/6′-H_e), 1.78 (m, 3H, 3′-H_a/4′-H_a/5′-H_a), 1.45 (m, 3H, 3′-H_e/4′-H_e/5′-H_e), [^{13}C]NMR ($CDCl_3$): 154.9 (C-2), 142.4 (C-4/C-6), 132.7 (C-5), 114.3/113.5 (C-1/C-3/C-7/C-8), 45.6 (C-1′), 30.1 (C-2′/C-6′), 26.6 (C-3′/C-5′), 25.1 (C-4′)

Compound ***10***

Colourless crystals, melting point 178 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 230 (4.8); 306 (3.3); 317 (3.4), GLC-MS (methanol-cI): $m/z = 361$ (100%); 363 (76.1%); 365 (10.1%) $[M+H]^+$, [^{1}H]NMR (323 K, $CDCl_3$): 3.62 (tt nr, 2H, 1″-H/1‴-H), 2.26, m (4H, 2″-H_a/6″-H_a/2‴-H_a/6‴-H_a), 1.93 (m, 4H, 2″-H_e/6″-H_e/2‴-H_e/6‴-H_e), 1.78/1.70 (m/m, 2H/4H, 3″-H_a/4″-H_a/5″-H_a/3‴-H_a/4‴-H_a/5‴-H_a), 1.42 (m, 6H, 3″-H_e/4″-H_e/5″-H_e/3‴-H_e/4‴-H_e/5‴-H_e), [^{13}C]NMR ($CDCl_3$): 154.3 (C-4/C-6), 142.0 (C-2), 134.1 (C-5), 114.4/112.4 (C-1/C-3/C-7/C-8), 43.2 (C-1″/C-1‴), 29.2 (C-2″/C-6″/C-2‴/C-6‴), 26.7 (C-3″/C-5″/C-3‴/C-5‴), 25.2 (C-4″/C-4‴)

Compound ***11***

Colourless crystals, melting point 155 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 230 (4.8); 303 (3.3); 315 (3.4), GLC-MS (methanol-cI): $m/z = 361$ (100%); 363 (66.5%); 365 (9.2%) $[M+H]^+$, [^{1}H]NMR (323 K, $CDCl_3$): 3.59 (tt, 1H, 1″-H, $^3J_{a,a}$ (1″-H; 2″-H_a/6″-H_a): 12.2 Hz), $^3J_{a,e}$ (1″-H; 2″-H_e/6″-H_e): 3.2 Hz), 3.39 (tt, 1H, 1′-H, $^3J_{a,a}$ (1′-H; 2′-H_a/6′-H_a): 12.4 Hz), $^3J_{a,e}$ (1′-H; 2′-H_e/6′-H_e): 3.3 Hz), 2.30 (m, 4H, 2′-H_a/6′-H_a/2″-H_a/6″-H_a), 1.77 (m, 4H, 2′-H_e/6′-H_e/2″-H_e/6″-H_e), 1.77/1.68 (m/m, 2H/4H, 3′-H_a/4′-H_a/5′-H_a/3″-H_a/4″-H_a/5″-H_a), 1.43 (m, 6H, 3′-H_e/4′-H_e/5′-H_e/3″-H_e/4″-H_e/5″-H_e), [^{13}C]NMR (373 K, d_6-DMSO): 154.4/154.1 (C-2/C-4), 141.6 (C-6), 132.2 (C-5), 115.6/114.4/113.1 (C-1/C-3/C-7/C-8), 44.9 (C-1′/C-1″), 29.8/28.6/26.6/25.35/25.27 (C-2′/C-3′/C-4′/C-5′/C-6′/C-2″/C-3″/C-4″/C-5″/C-6″)

Compound ***12***

Colourless crystals, melting point 262 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 229 (4.9); 301 (3.3); 312 (3.4), GLC-MS (methanol-cI): $m/z = 409$ (100%); 411 (27.5%) $[M+H]^+$, [^{1}H]NMR (323 K, $CDCl_3$): 3.65 (tt nr, 2H, 1″-H/1‴-H), 3.44 (bt, 1H, 1′-H, $^3J_{a,a}$ (1′-H; 2′-H_a/6′-H_a): ≈11 Hz), 2.35 (m, 6H, 2′-H_a/6′-H_a/2″-H_a/6″-H_a/2‴-H_a/6‴-H_a), 1.90 (m, 6H, 2′-H_e/6′-H_e/2″-H_e/6″-H_e/2‴-H_e/6‴-H_e), 1.71 (m, 9H, 3′-H_a/4′-H_a/5′-H_a/3″-H_a/4″-H_a/5″-H_a/3‴-H_a/4‴-H_a/5‴-H_a), 1.43 (m, 9H, 3′-H_e/4′-H_e/5′-H_e/3″-H_e/4″-H_e/5″-H_e/3‴-H_e/4‴-H_e/5‴-H_e), [^{13}C]NMR (323 K, $CDCl_3$): 154.9/153.5 (C-2/C-4/C-6), 133.4 (C-5), 116.7/111.4 (C-1/C-3/C-7/C-8), 44.6/43.3 (C-1′/C-1″/C-1‴), 29.6/28.9/27.0/26.9/25.5/25.3 (C-2′/C-3′/C-4′/C-5′/C-6′/C-2″/C-3″/C-4″/C-5″/C-6″/C-2‴/C-3‴/C-4‴/C-5‴/C-6‴)

Compound ***13***

Colourless crystals, melting point 57 °C, UV/VIS (hexane) λ_{max} [nm], (log ε): 230 (4.8); 308 (3.2); 320 (3.3), GLC-MS (methanol-cI): $m/z = 315$ (100%); 317 (98.2%); 319 (26.6%); 321 (2.8%) $[M+H]^+$, [^{1}H]NMR (323 K, $CDCl_3$): 3.75 (m, 1H, 2″-H), 2.03 (dddd, 1H, 3″-H_A, ^2J(3″-H_A; 3″-H_B): (−) 13.8 Hz, ^3J(3″-H_A; 2″-H)/^3J(3″-H_A; 4″-H_C)/^3J(3″-H_A; 3″-H_D): 5.6/8.2/9.9 Hz), 1.90 (m, 1H, 3″-H_B), 1.49 (d, 3H, 1″-H_3, ^3J(1″-H_3; 2″-H): 7.3 Hz), 1.33/1.12 (m/m, 3H/1H, 4″-H_C/4″-H_D/5″-H_2), 0.87 (t, 3H, 6″-H_3, ^3J(6″-H_3; 5″-H_2): 7.2 Hz), [^{13}C]NMR (323 K, $CDCl_3$): 156.0 (C-4), 142.0/140.8 (C-2/C-6), 134.1 (C-5), 115.3/113.2/112.0 (C-1/C-3/C-7/C-8), 38.5 (C-3″), 33.9 (C-2″), 30.3 (C-4″), 22.5 (C-5″), 18.2 (C-1″), 13.8 (C-6″)

REFERENCES

1 Schwack W, Photochemical reactions of pesticides with constituents of plant cuticles. 1. Model reactions of DDT and methoxychlor with cyclohexene. *Z Lebensm Unters Forsch* **179**:389–393 (1984).

2 Schwack W, Photoinduced additions of pesticides to biomolecules. 2. Model reactions of DDT and methoxychlor with methyl oleate. *J Ag Fd Chem* **36**:645–648 (1988).

3 Schwack W, Bourgeois B and Walker F, Fungicides and photochemistry–photodegradation of the dicarboximide fungicide procymidone. *Chemosphere* **31**:4033–4040 (1995).

4 Schwack W, Bourgeois B and Walker F, Fungicides and photochemistry: photodegradation of the dicarboximide fungicide vinclozolin. *J Ag Fd Chem* **43**:3088–3092 (1995).

5 Hartmann M Verhalten und Photoreaktivität der Azolfungizide Penconazol und Triadimefon auf Fruchtoberflächen – Auswirkung der Photochemie auf das Weinaroma. *PhD thesis,* University of Karlsruhe, Shaker, Aachen (1994).

6 Hahn A, Frimmel F, Haisch A, Henkelmann G and Hock B, Immunolabelling of atrazine residues in soil. *Z Pflanzenemaehr Bodenkd* **155**:203–208 (1992).

7 Ulrich P, Weller M and Niessner R, Immunological detection of triazine pesticides bound to soil humic acids (bound residues). *Fresenius J Anal Chem* **354**:352–358 (1996).

8 Sohn G, Sautter C and Hock B, Fluorescence immunolocalization of bound atrazine residues in plant tissue. *Planta* **181**:199–203 (1990).

9 Zorn H, Verhalten und Reaktivität des Fungicids Chlorothalonil auf Fruchtoberflächen unter besonderer Beachtung der photochemischen Bildung gebundener Rückstände. *PhD thesis,* University of Hohenheim, Shaker, Aachen, (1997).

10 Heilmann W, Battershell R, Pyne W, Goble P and Magee T, Synthesis and antiinflammatory evaluation of substituted isophthalonitriles, trimesonitriles, benzonitriles and terephthalonitriles. *J Med Chem* **21**:906–913 (1978).

11 Bersch B, Starck JP, Milon A, Nakatani Y and Ourisson G, Total synthesis of perdeuterated phospholipids. *Bull Soc Chim Fr* **130**:575–583 (1993).

12 Yelton DE, Diamond BA, Kwan S-P and Scharff MD, Fusion of mouse myeloma and spleen cells. *Curr Top Microbiol Immunol* **81**:1–7 (1978).
13 Hawkes R, Niday E and Gordon J, A dot-immunobinding assay for monoclonal and other antibodies. *Anal Biochem* **119**:142–147 (1982).
14 Schönherr J and Riederer M, Plant cuticles sorb lipophilic compounds during enzymatic isolation. *Plant Cell and Environment.* **9**:459–466 (1986).
15 Schynowski F, Untersuchungen der pflanzlichen Cuticula als Matrix photochemischer Reaktionen von Pestiziden. *PhD thesis,* University of Hohenheim, Shaker, Aachen (1996).
16 Caux P-Y, Kent RA and Fan GT, Environmental fate and effects of chlorothalonil: A Canadian perspective. *Crit Rev Environ Sci Tech* **26**:45–93 (1996).
17 Hock B, Immunochemical assay of environmental pollutants using pesticides as an example, in *Immunochemical Detection of Pesticides*, Deutsche Forschungsgemeinschaft. Research Report, Verlag Chemie, Weinheim (1995).